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Effects of combined exposures of fluoranthene and polyethylene or polyhydroxybutyrate microplastics on oxidative stress biomarkers in the blue mussel (*Mytilus edulis*)

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Running title: Bioplastics as a vector for environmental pollutants

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ABSTRACT

A growing interest in developing and commercialization of new ecofriendly plastic polymers is occurring attributed to the impact of marine plastics debris and microplastics that result from the degradation of oil-based polymers as these substances adversely affect ecosystem health. Recently, polyhydroxybutyrate (PHB) has become of interest due its biodegradability and physicochemical properties. However, biological consequences resulting from bioplastics exposure remains to be determined. Further, few data are apparently available regarding the potential for bioplastics to act as a vector for exogenous chemicals in the environment. The aim of the study was to compare the effects of polyethylene (PE MPs) and polyhydroxybutyrate (PHB MPs) microplastics administered alone or in combination with fluoranthene (Flu) on detoxifying enzymes in digestive glands and gills of *Mytilus edulis*. Blue mussels were exposed for 96hr to 8 experimental groups: control, Flu-only, PE MPs-only, PHB MPs-only, PE MPs-Flu co-exposure, PHB MPs-Flu co-exposure, Flu-incubated PE MPs and Flu-incubated PHB MPs. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx), glutathione S-transferase (GST) and glutathione reductase (GR) were found to be significantly susceptible to Flu and plastics in both tissues. Interestingly, single exposure to PHB MPs led to decreased levels of CAT and GST in gills, SOD in digestive glands and SeGPx in both tissues. In co-exposure and incubation treatments, biochemical responses were generally comparable with those exerted by PE MPs or PHB MPs only, suggesting an apparent absence of combined effects with pollutant. Data demonstrate the ecotoxicological impact of bioplastics materials on digestive glands and gills of *Mytilus edulis*.

Keywords: biodegradable polymer, biomarker profile, bioplastics, blue mussels, microplastic

Introduction

Oil-based plastic debris and subsequently the microplastics (MPs) formed by their degradation are one of the current major environmental concerns. These plastics are defined chemically as organic or semi-organic materials with long chains (macromolecules) and high molecular weight, generated by the polymerization of monomers extracted from oil or gases (Cole et al., 2011). Once in seawater, microplastics were shown to be ingested by aquatic species which resulted in a number of deleterious effects (Guzzetti et al., 2018; Alimba and Faggio, 2019) . The color, density, shape and size of MPs often leads marine organisms to mistake MPs for food items and the consequences of this includes blockage of the digestive system and false feeling of satiation (Wright et al., 2013). In addition, MPs may act as a vehicle for transport of several contaminants dissolved in the aquatic environment (Andrady, 2011). This 'vector effect' (Syberg et al., 2015), suggests that oil-based MPs may trap and transport environmental pollutants including persistent organic pollutants (POPs) or metals (Oliveira et al., 2013; Avio et al., 2015; Khan et al., 2015; Magara et al., 2018). The possibility that plastics might then transport the adhered contaminants into aquatic biota is currently being debated (Koelmans et al., 2016), and lab investigations into the vector effect remain ongoing.

There is a mounting interest in "green materials" and a focus on development and production of biodegradable polymers (Mohanty et al., 2002). In contrast to oil-based polymers, these biopolymers are derived from bioprocesses, using renewable resource in bio-refineries. Due to their mechanical and physicochemical characteristics, these biodegradable polymers may be considered as an eco-friendly substitutes to plastic (Anjum et al., 2016). In recent years, polyhydroxybutyrate (PHB) has received growing interest for its characteristics similar to oil-based plastic (Dacosta et al., 2016). Polyhydroxybutyrate is water insoluble, but highly biodegradable biopolymer, relatively resistant to hydrolytic degradation which is extracted with chloroform from bacterial cultures grown on carbohydrates. PHB (1) displays low oxygen permeability and reliable thermoplasticity with poor mechanical properties, (2) high crystallinity degree and (3) is optically active with a mass about approximately 0.5×10^6 Da (Anjum et al., 2016). However, although PHB is currently available commercially, especially as main component of biodegradable market bags, to the best of our knowledge little is known regarding the environmental impact or effects on aquatic organisms. By sharing many characteristics with oil-based plastics, it is feasible that in the aquatic environment, bioplastic undergoes modifications, resulting in generation of MPs as is the case for oil-based polymers (Andrady, 2011). If due to environmental and societal pressures a move is made towards use of bioplastics, then it is vital to gather information on the influence of these bioplastics on the ecosystem health.

Oxidative stress results from an imbalance between pro-oxidants such as reactive oxygen species (ROS), and the protective antioxidant system. Mechanisms that involve glutathione (GSH), and the related antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidases (SeGPx and GPx) represent important protective metabolic pathways which are used as biomarkers related to pollutant induced oxidative stress. Glutathione (GSH) in its reduced form is an important non-enzymatic scavenger of oxyradicals, involved in the metabolism of toxic compounds and endogenous substances (Meister and Anderson, 1983). Both SOD and CAT enzymes catalyze the breakdown of ROS-generating O_2^- and H_2O_2, respectively and are key components within the primary defense system against oxidative stress-induced damage. SeGPx enzyme reduces either hydrogen peroxide or organic peroxides and GPx enzyme affects organic hydroperoxides. Polycyclic aromatic hydrocarbons (PAHs) , of which fluoranthene (Flu) is often used as a model contaminant (and in addition a priority aquatic pollutant (Directive 2008/105/EC)), are known to initiate oxidative stress which was assessed by these aforementioned biomarkers (Cheung et al., 2001). The oxidative stress biomarkers employed in the present study were utilized to determine the impact of a range of xenobiotics on aquatic organisms (Al kaddissi et al., 2012; 2016; Cozzari et al., 2015; Elia et al., 2017a; 2017 b; 2018; Magara et al., 2018). Previous studies utilizing these biomarkers to elucidate the

MP vector effect demonstrated a perturbation of intracellular antioxidant defenses (Oliveira et al., 2013; Avio et al., 2015; Magara et al., 2018).

Previously Magara et al. (2018) investigated the influence of polyethylene microplastic beads (PE MPs) on the accumulation and associated oxidative stress responses attributed to Flu in blue mussels, *Mytilus edulis*. The mussels were exposed to 4 treatment groups: Flu-only, MP-only, Flu and MP co-exposure, and Flu-incubated MP. Individual contaminant exposures to Flu or MP resulted in varying responses, but co-exposures and incubated treatments (i.e. Flu was sorbed to plastic surfaces prior to exposure) did not induce additive or synergistic responses. Further, MP-only exposure appeared to be capable of eliciting direct effects on the oxidative stress system as evidenced by enhanced activities of CAT and GPx (Magara et al., 2018).

Thus, the present study was designed to investigate the potential impact of PHB microplastics as a single contaminant and in combination with Flu on the oxidative stress system of blue mussel (*Mytilus edulis*), a well-regarded test species for MPs (Avio et al., 2015; Paul-Pont et al., 2016; Sahlmann et al. 2017; Von Moos, 2012). PE MPs, as an example of oil-based MPs, were employed as a comparison to PHB MPs. The aim of the study was to examine the comparative changes on oxidative stress biomarkers levels induced by oil based (PE) MPs and 'new' bioplastic PHB MPs as both single contaminants and as potential vectors for PAHs.

Materials and methods

Mussels collection and maintenance

Blue mussel (*Mytilus edulis*) were collected in June, 2016, in Vellerup Vig – Isefjord, N 55°40.6'; E 11°48.7', in an non-polluted deep inlet of Baltic Sea near Roskilde (Denmark). The mussels were maintained in a temperature-controlled room (10 ± 1°C). All specimens were placed in tanks (size 39 x 21 x 25 cm), filled with 10 L of field water. Field water was gradually replaced with lab water maintaining similar salinity (20 salinity). Mussels were fed for the following three weeks twice weekly. A 16:8 hr light/dark photoperiod was maintained throughout.

Chemicals and preparation

A primary stock solution of Flu (Fluka Chemika, Steinheim, Switzerland) was prepared at 1 mg/ml in acetone. PE MPs were purchased from Cospheric LLC (Santa, Barbra, CA, USA) and PHB beads provided by Prodotti Gianni S.R.L. (Milano, Italy). Both polymers were white in color and similar in size (10–90 μm). Both MPs were treated with 0.1% Tween80 to enable dispersion in water column (Khan et al., 2015).

To prepare each exposure treatment accurately a weight to MP number ratio was determined for both PE and PHB particles. The weight associated to 1×10^6 MPs was weighed out for each treatment group containing MPs. The MPs were dispersed in 5 ml of 0.1% Tween80 solution and shaken at 150 rpm overnight. The MPs were filtered through 1 μm nylon mesh and resuspended in 200 ml 20 salinity water and kept shaking until the start of the experiment (150 rpm). MPs that required incubation with Flu were resuspended into 20 ml acetone to which the appropriate amount of Flu stock was added to yield the correct final concentration of 100 μg/L when made up to the final exposure solution. After an initial mixing, dispersions were left under a fume hood for the acetone to evaporate and the Flu sorb to the PE MPs. This method was adopted from use with other particulate contaminants (Al-Subiai et al., 2012). When complete dryness was achieved overnight the Flu-incubated MPs were resuspended in 200 ml 20 salinity water and placed with the other dispersions on the shaking table overnight.

Mussels exposure

Mussels were exposed for 96 hr to 8 treatment groups as follows, (A) Control (no added contaminants), (B) Flu only, (C) PE MPs only, (D) PHB MPs only, (E) PE MPs-Flu co-exposure, (F) PHB MPs-Flu co-exposure, (G) Flu-incubated PE MPs and (H) Flu-incubated PHB MPs (n=4 per treatment). The Flu and MP concentrations across all treatments were 100 μg/L and 1000 MPs/ml, respectively. Although the concentrations exceeded environmental realistic conditions , the aim of this study was to understand mechanistic responses that are more easily captured at higher exposure levels. The concentrations used are consistent with previous studies (Al-Subiai et al.,2012; Cole et al., 2016; Magara et al., 2018). Acetone and Tween80 were consistently added across treatments to prevent the confounding influence of solvent and detergent. Ninety-six hr mussel exposures were conducted at 10±1°C and a 16/8 hr light/dark photoperiod. Mussels were individually exposed in aerated beakers (250 ml) filled with 200 ml treatment solution. Four samples of two pooled mussels were exposed per treatment group.

To start the experiment, each 200 ml dispersion was made up to 1 L with 20 salinity water and vigorously stirred to ensure homogenous distribution of MPs in the water column. The 1 L dispersion was then divided into 5 200 ml units again ensuring a homogenous distribution of MPs between beakers. The appropriate amount of Flu stock was added to the Flu-only and co-exposures groups prior to the start of the experiment. Post exposure (96 hr), mussels were removed and rinsed thoroughly under 20 salinity water. Gill and digestive gland tissues were extracted from each individual and placed in separate pre-weighed and pre-labelled vials. Tissues for biomarkers and Flu concentration analysis were stored at -80ºC and -20º C, respectively. No mortalities were recorded during the experiments. .

Biochemical analysis

The analysis of biomarkers is described in detail elsewhere (Cozzari et al., 2015; Magara et al., 2018). Briefly, total GSH levels as well as activities of SOD, CAT, GPx and SeGPx were measured in cytosolic fraction of gills and digestive gland spectrophotometrically (Varian Cary 50 spectrophotometer at 25ºC). Protein concentrations in the cytosol were determined according to Lowry et al. (1951) and used to normalize enzyme levels. All analyses were performed in triplicate along with blank samples (buffer and reagents only). These absorbance values were subtracted from those of the sample. SOD activity was determined according to an established method (McCord and Fridovich, 1969). Catalase levels were measured following the decrease in absorbance due to H_2O_2 consumption at 240 nm (Greenwald, 1985). GPx activities (SeGPx and total GPx) were determined following the oxidation of nicotinamide adenine dinucleotide phosphate reduced form (NADPH) at 340 nm (Lawrence and Burk, 1976). GR activity was measured in 100 mM NaH2PO4 + Na2HPO4 buffer, pH 7, 1 mM oxidized glutathione (GSSG), and 60 μM NADPH; the decrease in absorbance due to the oxidation of NADPH was measured at 340 nm (Chung et al. 1991). GST activity was determined in 100 mM NaH2PO4 + Na2HPO4 buffer, pH 6.5, 1 mM GSH, 1 mM 1-chloro-2,4 dinitrobenzene (CDNB) as substrates and sample. Formation of the conjugate with GSH was read at 340 nm (Habig et al. 1974). Total glutathione (GSH+2GSSG) was measured following homogenization in 5% sulphosalicilic acid with 4mM EDTA by the GR recycling assay at 412 nm (Akerboom and Sies, 1981).

Statistical analysis

Data are reported as mean values ± the standard deviation (SD). Levene's test for normal distribution was performed on datasets prior to statistical analysis. A one-way ANOVA with Bonferroni’s multiple comparison test was used to investigate differences between treatment groups (GraphPad Prism software). Statistical significance was set at $P < 0.05$.

Results

In the gills, CAT activity levels significantly decreased in all treatment groups (up to 80%), except for PE MPs only exposure compared to control (Figure 1C). The activity of SeGPx was markedly lower (up to 90%) through the treatments (Figure 2C). GST levels in the treated groups were significantly lower than control (up to 50%), except for Flu-incubated PHB MPs (Figure 3A). No marked changes of SOD, GPx and GR activities were noted for all treatment compared to respective controls (Figures 1A, 2A, 3C).

In the digestive glands, SOD activity was significantly reduced (up to 50%) in all treatments when compared to control with the exception of the group incubated with to PE MPs only (Figure 1B). CAT activity was markedly decreased similar to SOD (up to 60%)., However, no significant differences were found in mussels exposed to PHB MPs only and Flu-incubated PHB MPs (Figure 1D). Total GPx markedly fell (60%) following Flu exposure (Figure 2B). Levels of SeGPx activity were significantly reduced compared to control (up to 50%), except for PE MPs only and Flu-incubated PHB MPs groups (Figure 2D). GST activity peaked (1.5 fold higher) in Flu exposed group (Figure 3B), whereas GR activity levels showed increasing trends in all treated groups. The GR activity was markedly higher following PE MPs only exposure (1 fold), PE MPs-Flu co-exposure (1.2 fold) and Flu-incubated PHB MPs treatment (1 fold, Figure 3D).

Discussion

The present study provides the first experimental evidence of stress-related effects initiated by novel bio-microplastic exposure, either as a single contaminant or in combination with Flu, in the blue mussel *Mytilus edulis*. The comparison was conducted with the more traditional oil-based plastic polyethylene. Flu and both PE and PHB MPs modified the antioxidant responses in both gills and digestive glands. The present results are largely in agreement with those previously reported for Flu and PE MPs, both in single or combined exposures (Magara et al., 2018).

PE MPs altered the levels of several of the tested biomarkers. Although previous studies showed that oil-based plastic led to enhanced antioxidant activities in copepods (Jeong et al., 2017) and fish (Barboza et al., 2018), our results are in agreement with studies on mussels, showing a significant inhibition of CAT, SeGPx and GST activity following oil-based MPs mussel exposure (Avio et al., 2015; Paul-Pont et al., 2016). A decrease of CAT and GPx activities in digestive glands and of SeGPx and GST in gills exposed to PE MPs may be related to the size and shape of microplastics, that play a key role in initiation of biological changes (Browne et al., 2008; Von Moos, 2012; Avio et al., 2015). Small polystyrene plastic particles may be translocated from the gut to the circulatory system of *Mytilus edulis* and subsequently retained suggesting that these substances y might reach crucial organs such as the heart or hepatic tissues to produce adverse effects (Browne et al., 2008). It is well known that oil-based microplastics may accumulate within organisms and induce tissue abrasions, as evidenced by histological changes in digestive cells and triggered inflammatory responses, formation of granulocytomas and lysosomal destabilization (Avio et al., 2015; Von Moos, 2012). Therefore, in this scenario, the imbalance of antioxidant defense markers might be expected and might be attributed to physical damage induced by MPs enabling further accumulation of reactive products. Further, in the present study increased GR activity in digestive glands might reflect the demand for GSH as defense mechanism against oxidative stress induced by oil-based MPs (Jeong et al., 2016).

The present study also carried out a co-exposure and incubation scenario of both PE and PHB MPs with Flu and reported information on oxidative stress related to the simultaneous exposure to compounds. In this scenario, a mussel experimental group exposed to Flu-only was used as positive control. Previously Pan et al (2005) found that at high benzo(k)fluoranthene, a PAH, concentrations the activity of antioxidant enzymes was diminished in the scallop *Chlamys farreri* in a time-dependent manner. Our findings that the marked decrease of SOD activity levels and both GPx in digestive glands, as well as SeGPx in gills and CAT activity in both tissues indicate that this Flu may exert detrimental effects when administered alone resulting in a severe oxidative stress in *Mytilus edulis*. The impairment of antioxidant biomarkers levels may be attributed to a chemical-mediated damage. Indeed, the enhanced GST activity measured in digestive glands might represent an important outcome, since this indicate elevated of phase-II biotransformation metabolism. The significant induction of GST activity in mussels exposed to Flu-only may be attributed to production of specific substrates for GST by phase I enzymes, representing an effective defense line against Flu. Babson et al (1986) showed that rat liver microsomes converted Flu to trans-2,3-dihydrodiol as the major metabolite. Although data regarding Flu metabolites in blue mussels are lacking, it is suggested that GST induction may be related to production of this intermediate.

Biomarkers responses in digestive glands and gills of mussels exposed to both Flu and PE or PHB MPs (co-exposure and incubated treatment) generally followed a trend similar to PAH alone or the new plastic alone and no combined effects of these agents was apparent. The absence of combined effects in *Mytilus edulis* exposed to PE MPs and Flu is in agreement with our previous results (Magara et al., 2018), suggesting that MPs may act as a "sink" of environmental pollutants (Chua et al. 2014; Khan et al. 2015). Chua et al. (2014), showed that the concentration of polybrominated diphenyl ethers (PBDE) was lower in *Allorchestes compressa* co-exposed to microplastics then specimens treated with PBDE alone. Chua et al (2014) concluded that the presence of MPs may inhibit the uptake of PBDE, perhaps because this contaminant is strongly absorbed onto microplastics surface making it less bioavailable. It is already known that oil-based micropolymers have the propensity to aggregate in water (Alimi et al., 2018) . Previously Khan et al (2015) found in zebrafish a decrease in silver (Ag) uptake by fish attributed to diminished contact between Ag and tissues following oil-based MP aggregation. Further, Magara et al. (2018) reported that Flu tissue concentrations were lower in *Mytilus edulis* exposed to both co-exposure with polyethylene and PAH compared to specimens treated with Flu alone. The absence of combined effects of PE MPs and Flu in *Mytilus edulis* and previous results on Flu uptake in blue mussel (Magara et al., 2018), suggests that the interaction of tissues with Flu might be delayed by this aggregation mechanism exerted by microplastics.

Bioplastics are currently becoming the leading material for replacing oil-based polymers since it is more desirable than traditional plastic due to a propensity to biodegrade in the environment (Anjum et al., 2016). Recently Napper and Thompson (2019) demonstrated that biodegradable polymers may not undergo any substantial deterioration over a 3 year period in marine environment, but may be reduced in small fragments similar to oil-based plastics. Our data suggest that PHB MPs results in altered levels of some oxidative stress biomarkers similar to oil-based MPs. The fact that bio-microplastics may exert stress effects and the potential low biodegradability rate indicate a novel threat for the marine environment. Therefore, it is crucial to understand degradation processes of bioplastics in the environment, but also gathering knowledge regarding potential ecotoxicological impacts. Although the damage exerted by oil-based polymers is becoming increasingly understood, it is still necessary to investigate the possible consequences of bioplastics exposure both alone and in combination with other environmental pollutants present in the aquatic ecosystem.

Conclusions

The results of the present study demonstrated that PHB MPs modify the baseline levels of biomarkers related to oxidative stress in *Mytilus edulis*. These alterations were similar to those exerted by PE MPs treatments for some of the antioxidant biomarkers. Further, the responses measured in both co-treatments were similar to those noted with MP-alone exposures. Data indicated the absence of any combined effects produced by oil-based MPs or bioplastics MPs and Flu.

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Legend of Figures

Figure 1. SOD (A and B) and CAT (C and D) activity in gills (A and C) and digestive gland (B and D) of *Mytilus edulis* exposed to different treatment groups A-H as follows: (A) Control (no added contaminants), (B) Flu only, (C) PE MPs only, (D) PHB MPs only, (E) PE MPs-Flu co-exposure, (F) PHB MPs-Flu co-exposure, (G) Flu-incubated PE MPs and (H) Flu-incubated PHB MPs. Bars show mean values ± S.D. Different letters (a, b, c) indicate statistical significant differences ($P < 0.05$**,** one-way ANOVA with post-hoc Bonferroni test). Note different scales on different x-axes.

Figure 2. GPx (A and B) and SeGPx (C and D) activity in gills (A and C) and digestive gland (B and D) of *Mytilus edulis* exposed to different treatment groups A-H as follows: (A) Control (no added contaminants), (B) Flu only, (C) PE MPs only, (D) PHB MPs only, (E) PE MPs-Flu co-exposure, (F) PHB MPs-Flu co-exposure, (G) Flu-incubated PE MPs and (H) Flu-incubated PHB MPs. Bars show mean values ± S.D. Different letters (a, b, c) indicate statistical significant differences ($P < 0.05$**,** one-way ANOVA with post-hoc Bonferroni test). Note different scales on different x-axes.

Figure 3. GST (A and B) and GR (C and D) activity in gills (A and C) and digestive gland (B and D) of *Mytilus edulis* exposed to different treatment groups A-H as follows: (A) Control (no added contaminants), (B) Flu only, (C) PE MPs only, (D) PHB MPs only, (E) PE MPs-Flu co-exposure, (F) PHB MPs-Flu co-exposure, (G) Flu-incubated PE MPs and (H) Flu-incubated PHB MPs.

Bars show mean values ± S.D. Different letters (a, b, c) indicate statistical significant differences ($P < 0.05$**,** one-way ANOVA with post-hoc Bonferroni test).

~~Declarations of interest: none.~~

Effects of combined exposures of fluoranthene and polyethylene or polyhydroxybutyrate microplastics on oxidative stress biomarkers in the blue mussel (*Mytilus edulis*)

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Running title: Bioplastics as a vector for environmental pollutants

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ABSTRACT

A growing interest in developing and commercialization of ~~ing~~ new ecofriendly plastic polymers is occurring attributed ~~owing~~ to the impact of marine plastics debris and microplastics that result from the degradation of oil-based polymers as these substances adversely affect ecosystem health. Recently, polyhydroxybutyrate (PHB) has become of interest due its biodegradability and physico~~al~~ chemical properties. However, biological consequences~~impacts~~ resulting from bioplastics exposure remains to be determined.~~is yet unknown.~~ ~~Moreover~~Further, few data are apparently available regarding ~~nothing is known about~~ the potential for bioplastics to act as a vector for exogenous chemicals in the environment. The aim of the study was to compare the effects of polyethylene (PE MPs) and polyhydroxybutyrate (PHB MPs) microplastics administered alone~~singularly~~ or in combination with fluoranthene (Flu) on detoxifying enzymes in digestive glands and gills of *Mytilus edulis*. Blue mussels were exposed for 96hr to 8 ~~eight~~ experimental groups: control, Flu-only, PE MPs-only, PHB MPs-only, PE MPs-Flu co-exposure, PHB MPs-Flu co-exposure, Flu-incubated PE MPs and Flu-incubated PHB MPs. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx), glutathione S-transferase (GST) and glutathione reductase (GR) were found~~have proved~~ to be significantly susceptible to Flu and plastics in both tissues. Interestingly, single exposure to PHB MPs ~~single exposure~~ led to decreased levels of CAT and GST in gills, SOD in digestive glands and SeGPx in both tissues ~~biomarkers biomarkers STATE INCREASE OR DECREASE?in SOD, CAT, etc.impairments~~. In co-exposure and incubation treatments, biochemical responses were generally comparable with those exerted by PE MPs or PHB MPs only, suggesting an apparent absence of ~~no~~ combined effects with pollutant. ~~The present findings are amongst the first to describe~~ Data demonstrate the ecotoxicological ~~potential~~ impact of bioplastics materials on digestive glands and gills of *Mytilus edulis*.

Keywords: biodegradable polymer, biomarker profile, ~~-~~bioplastics, blue mussels, microplastic

Introduction

Oil-based plastic debris and subsequently the microplastics (MPs) formed by their degradation are one of the current major ~~biggest~~ environmental concerns. ~~issues~~ ~~of the day.~~ These plastics are defined chemically as organic or semi-organic materials with long chains (macromolecules) and high molecular weight, generated by the polymerization of monomers extracted from oil or gases (Cole et al., 2011). Once in seawater, microplastics were ~~have been~~ shown to be ingested by aquatic species which resulted in ~~can~~ ~~lead to~~ a number of deleterious effects (Guzzetti et al., 2018; Alimba and Faggio, 2019) ~~(NEED REF??)~~. The color, density, shape and size of MPs often leads marine organisms to mistake MPs for food items and the consequences of this includes~~,~~ blockage of the digestive system and false feeling of satiation (Wright et al., 2013). In addition, ~~, and also~~ MPs may act as a vehicle for ~~the~~ transport of several contaminants dissolved in the aquatic environment (Andrady, 2011). This ‘vector effect’ (Syberg et al., 2015), suggests that oil-based MPs may trap and transport environmental pollutants including~~, such as~~ persistent organic pollutants (POPs) or metals (Oliveira et al., 2013; Avio et al., 2015; Khan et al., 2015; Magara et al., 2018). The possibility that plastics might ~~can~~ then transport the adhered contaminants into aquatic biota is currently being ~~still~~ debated (Koelmans et al., 2016), and ~~but~~ lab~~oratory~~ investigations into the vector effect remain ongoing.

There is a mounting interest in “green materials” and a focus on ~~the~~ development and production of biodegradable polymers (Mohanty et al., 2002). In contrast to~~Unlike~~ oil-based polymers, these biopolymers are derived from bioprocesses, using renewable resource in bio-refineries.~~, and~~ ~~d~~Due to their mechanical and physicochemical ~~-physical~~ characteristics, these biodegradable polymers may be considered as an eco-friendly substitutes to plastic (Anjum et al., 2016). In recent years, polyhydroxybutyrate (PHB) has received ~~ing~~ growing interest for its characteristics similar to oil-based plastic ~~(NEED REF??)~~(Dacosta et al., 2016). ~~It~~ Polyhydroxybutyrate is ~~a~~ water insoluble, but highly biodegradable biopolymer, relatively resistant to hydrolytic degradation which ~~. It~~ is extracted with chloroform from bacterial cultures ~~which~~ ~~is~~ grown on carbohydrates. PHB (1) displays~~has~~ low oxygen permeability and reliable ~~good~~ thermoplasticity with poor mechanical properties, (2) high crystallinity degree and (3) is optically active~~,~~ with a mass about approximately 0.5×10^6 Da (Anjum et al., 2016). However, although PHB is currently available commercially, especially as main component of biodegradable market bags, to the ~~our~~ best of our knowledge little is known regarding ~~about~~ ~~it is~~ the environmental impact or effects on aquatic organisms. By sharing many characteristics with oil-based plastics, it is feasible that in the aquatic environment, bioplastic undergoes modifications, resulting in ~~leading to~~ generation of MPs as is the case for oil-based polymers (Andrady, 2011). If due to environmental and societal pressures a move is made towards use of bioplastics, then it is vital to gather information on the influence of these bioplastics on the ecosystem health.

Oxidative stress results from an imbalance between pro-oxidants~~,~~ such as reactive oxygen species (ROS), and the protective antioxidant system. Mechanisms that involve glutathione (GSH), and the related antioxidant enzymes ~~s~~ superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidases (SeGPx and GPx) represent important protective metabolic pathways which ~~and~~ are used as biomarkers related to pollutant induced oxidative stress. Glutathione (GSH) in its reduced form is an important non-enzymatic scavenger of oxyradicals, ~~and is~~ involved in the metabolism of toxic compounds and endogenous substances (Meister and Anderson, 1983). Both SOD and CAT enzymes catalyze the breakdown of ROS-generating~~causing~~ O_2^- and H_2O_2, respectively and are key components within the primary defense system against oxidative stress-induced damage. SeGPx enzyme ~~can~~ reduces either hydrogen peroxide or organic peroxides and GPx enzyme affects organic hydroperoxides. Polycyclic aromatic hydrocarbons (PAHs), of which fluoranthene (Flu) is often used as a model contaminant (and in addition a priority aquatic pollutant (Directive 2008/105/EC)), are known to initiate~~generate~~ oxidative stress which was ~~has been~~ assessed by these aforementioned biomarkers (Cheung et al., 2001). The oxidative stress biomarkers employed ~~used~~ in the present study~~,~~ were~~have been~~ utilized to determine ~~assess~~ the impact of a range of xenobiotics on aquatic organisms (Al kaddissi et al., 2012;~~,~~ 2016; Cozzari et al., 2015; Elia et al., 2017a~~,~~; 2017 b;~~,~~ 2018; Magara et al., 2018). Previous studies utilizing these biomarkers to elucidate the MP vector effect ~~have~~ demonstrated a perturbation of intracellular antioxidant defenses (Oliveira et al., 2013; Avio et al., 2015; Magara et al., 2018).

~~Our~~ ~~p~~ Previously ~~study~~ ~~(~~Magara et al~~.,~~(2018) investigated the influence of polyethylene microplastic beads (PE MPs) on the accumulation and associated oxidative stress responses attributed to ~~fluoranthene (~~Flu~~)~~ in blue mussels, *Mytilus edulis*. The mussels were exposed to 4 ~~four~~ treatment groups: Flu-only, MP-only, Flu and MP co-exposure, and Flu-incubated MP. Individual contaminant exposures to Flu or MP resulted in~~led to~~ varying responses, but co-exposures and ~~WHAT IS THIS~~incubated~~ ???DO YOU MEAN *IN VITRO*?~~treatments (i.e. Flu was sorbed to plastic surfaces prior to exposure)~~exposures~~ did not induce ~~result~~ ~~in~~ additive or synergistic responses.~~effects.~~ ~~Moreover~~ Further, MP-only exposure appeared to be capable of eliciting direct effects on the oxidative stress system as evidenced ~~demonstrated~~ by ~~the~~ enhanced activities of CAT and GPx (Magara et al., 2018).

~~If due to environmental and societal concerns a move is made towards bioplastics, then it is vital to gather information on the impacts of the bioplastics. This includes the possibility of bioplastics as vectors of other pollutants.~~

Thus, the present study was designed to investigate the potential impact of PHB microplastics as a single contaminant and in combination with ~~fluoranthene (~~Flu~~), a representative PAH,~~ on the oxidative stress system of blue mussel (*Mytilus edulis*), a well-regarded test species for MPs (Avio et al., 2015; Paul-Pont et al., 2016; Sahlmann et al 2017; Von Moos, 2012). PE MPs, as an example of oil-based MPs, were employed as~~used~~ a comparison to ~~the~~ PHB MPs. The aim of the study was to examine the comparative changes on oxidative stress biomarkers levels induced by oil based (PE) MPs and 'new' bioplastic PHB MPs as both single contaminants and as potential vectors for PAHs.

~~SHIFT TO METHODSMussels were exposed for 96 h to seven treatment groups; Control (no added contaminants), Flu only, PE MPs only, PHB MPs only, PE MPs-Flu co-exposure, PHB MPs-Flu co-exposure, Flu-incubated PE MPs and Flu-incubated PHB MPs.~~ !!

~~The aim of the study was to investigate the comparative changes on oxidative stress biomarkers levels induced by oil based (PE) MPs and 'new' bioplastic PHB MPs as both single contaminants and as potential vectors for PAHs.~~

Materials and methods

Mussels collection and maintenance

Blue mussel (*Mytilus edulis*) were collected in June, 2016, in Vellerup Vig – Isefjord, N 55°40.6'; E 11°48.7', in an ~~un~~ non-polluted deep inlet of Baltic Sea near Roskilde (Denmark). The mussels were maintained ~~located~~ in a temperature-controlled room (10 ± 1°C). All specimens were placed in tanks (size 39 x 21 x 25 cm), filled with 10 L of field water. Field water was gradually replaced with lab~~oratory~~ water maintaining similar salinity (20 salinity). Mussels were fed for the following three weeks twice weekly. A 16:8 hr light/dark photoperiod was maintained throughout.

Chemicals and preparation

A primary stock solution of Flu (Fluka Chemika, Steinheim, Switzerland) was prepared at 1 mg/ml ~~L~~ in acetone. PE MPs were purchased from Cospheric LLC (Santa, Barbra, CA, USA) and PHB beads ~~were~~ provided by Prodotti Gianni S.R.L. (Milano, Italy). Both polymers were white in color and similar in size (10–90 μm). Both MPs were treated with 0.1% Tween80 to enable ~~allow~~ dispersion in water column (Khan et al., 2015).

To prepare each exposure treatment ~~correct~~ accurately a weight to MP number ratio was determined for both PE and PHB particles. The weight associated to 1 × 10^6 MPs was weighed out for each treatment group containing MPs. The MPs were dispersed in 5 ml of 0.1% Tween80 solution and shaken at 150 rpm overnight. The MPs were filtered through 1 µm nylon mesh and resuspended in 200 ml ~~L of~~ 20 salinity water and kept shaking until the start of the experiment (150 rpm). MPs that required incubation with Flu were resuspended into 20 ml ~~L of~~ acetone to which the appropriate amount of Flu stock was added to yield~~give~~ the correct final concentration of 100 µg/~~-~~L~~-1~~ when made up to the final exposure solution. After an initial mixing, dispersions were left under a fume hood for the acetone to evaporate and the Flu sorb to the PE MPs. This method was adopted from use with other particulate contaminants (Al-Subiai et al., 2012). ~~Having achieved~~ When complete dryness was achieved overnight the Flu-incubated MPs were resuspended in 200 ml~~L of~~ 20 salinity water and ~~were~~ placed with the other dispersions on the shaking table overnight.

Mussels exposure

Mussels were exposed for 96 hr to 8 ~~eight~~ treatment groups as follows, (A) Control (no added contaminants), (B) Flu only, (C) PE MPs only, (D) PHB MPs only, (E) PE MPs-Flu co-exposure, (F) PHB MPs-Flu co-exposure, (G) Flu-incubated PE MPs and (H) Flu-incubated PHB MPs (n=4 per treatment).~~(1) Control (no added contaminants), (2) Flu only, (3) PE MPs only, (4) PHB MPs only, (5) PE MPs-Flu co-exposure, (6) PHB MPs-Flu co-exposure, (7) Flu-incubated PE MPs and (8) Flu-incubated PHB MPs.~~ The Flu and MP concentrations across all treatments were ~~as~~ 100 µg/~~-~~L~~-1~~ and 1000 MPs/~~—~~ml~~L-1~~, respectively. Although the concentrations exceeded environmental realistic conditions ~~m~~, the aim of this study was to understand mechanistic responses that are more easily captured at higher exposure ~~concentration~~ levels. The concentrations used are consistent with previous studies (Al-Subiai et al.,2012; Cole et al., 2016; Magara et al., 2018). Acetone and ~~t~~Tween80 were consistently added across treatments to prevent the confounding influence ~~mpact~~ of solvent and detergent. Ninety-six hr ~~our~~ mussel exposures were conducted at 10±1°C and a 16/8 hr light/dark photoperiod. Mussels were individually exposed in aerated beakers (250 ml~~L~~) filled with 200 ml~~L of exposure~~treatment solution. ~~Five~~ Four samples of two pooled mussels were exposed per treatment group.

To start the experiment, each 200 ml ~~L~~ dispersion was made up to 1 L with 20 salinity water and vigorously stirred to ensure ~~the~~ homogenous distribution of MPs in the water column. The 1 L dispersion was then divided ~~split~~ into 5 ~~five~~ 200 ml units ~~L exposures,~~ again ensuring a homogenous distribution of MPs between beakers. The appropriate amount of Flu stock was added to the Flu-only and co-exposures groups prior to ~~before~~ the start of the experiment. Post exposure (96 hr), mussels were removed and rinsed thoroughly under 20 salinity water. Gill and digestive gland tissues were extracted ~~dissected~~ from each individual and placed in separate pre-weighed and pre-labelled vials. Tissues for biomarkers and Flu concentration analysis were stored at -80ºC and -20º C, respectively. No mortalities were recorded during the experiments. ~~osure~~.

Biochemical analysis

The analysis of biomarkers is described in detail elsewhere (Cozzari et al., 2015; Magara et al., 2018). Briefly, total GSH levels ~~glutathione,~~ as well as activities of SOD, CAT, GPx and SeGPx were measured in cytosolic fraction of gills and digestive gland ~~by~~ spectrophotometrically ~~analysis~~ (Varian Cary 50 spectrophotometer at 25ºC). Protein concentrations in the cytosol were determined according to Lowry et al. (1951) and ~~were~~ used to normalize enzyme levels. All analyses were performed in triplicate along with blank samples (buffer and reagents only). These absorbance values were subtracted from those of the sample. SOD activity was determined according to an established method (McCord and Fridovich, 1969). Catalase levels were measured following the decrease in absorbance due to H_2O_2 consumption at 240 nm (Greenwald, 1985). GPx activities (SeGPx and total GPx) were determined following the oxidation of nicotinamide adenine dinucleotide phosphate reduced form

(NADPH) at 340 nm (Lawrence and Burk, 1976). GR activity was measured in 100 mM NaH2PO4 + Na2HPO4 buffer, pH 7, 1 mM oxidized glutathione (GSSG), and 60 µM NADPH; the decrease in absorbance due to the oxidation of NADPH was measured at 340 nm (Chung et al. 1991). GST activity was determined in 100 mM NaH2PO4 + Na2HPO4 buffer, pH 6.5, 1 mM GSH, 1 mM 1-chloro-2,4 dinitrobenzene (CDNB) as substrates and sample. Formation of the conjugate with GSH was read at 340 nm (Habig et al. 1974). Total glutathione (GSH+2GSSG) was measured following homogenization in 5% sulphosalicilic acid with 4mM EDTA by the GR recycling assay at 412 nm (Akerboom and Sies, 1981).

Statistical analysis

Data are reported as mean values ± the standard deviation (SD). Levene's test for normal distribution was performed on datasets prior to statistical analysis. A one-way ANOVA with Bonferroni's multiple comparison test was used to investigate differences between treatment groups (GraphPad Prism software). Statistical significance was set at $P < 0.05$.

Results

In the gills, CAT activity levels significantly decreased in all treatment groups (up to 80%), except for PE MPs only exposure ~~when~~ compared to control (Figure 1C). The activity of SeGPx was markedly ~~consistently~~ lower (up to 90%) through the treatments (Figure 2C). GST levels in the treated groups were significantly lower than control (up to 50%), ~~unless~~ ~~exposed to~~except for Flu-incubated PHB MPs (Figure 3A). No marked changes of SOD, GPx and GR activities ~~y~~ were noted~~recorded~~ for all ~~the~~ treatment compared to respective~~the own~~ controls (Figures 1A, 2A, 3C).

In the digestive glands, SOD activity ~~concentrations~~ ~~were~~ was significantly reduced ~~consistently lowered~~ (up to 50%) in all treatments when compared to control with the exception of the group incubated with~~exposed~~ to PE MPs only (Figure 1B). CAT activity was markedly decreased ~~lowered~~

similar ~~ly~~ to SOD (up to 60%)., ~~but no statistically~~ However, no significant differences were found ~~recorded~~ in mussels exposed to PHB MPs only and Flu-incubated PHB MPs (Figure 1D). Total GPx markedly fell ~~dropped~~ (60%) following Flu exposure (Figure 2B). Levels of SeGPx activity were ~~reliably lower~~ significantly reduced compared to ~~than~~ control (up to 50%), except ~~unless~~ for PE MPs only and Flu-incubated PHB MPs groups (Figure 2D). GST activity peaked (1.5 fold higher) in Flu exposed group (Figure 3B), whereas GR activity levels showed increasing trends in all treated groups. ~~;~~ ~~t~~The GR activity was markedly higher following PE MPs only exposure (1 fold), PE MPs-Flu co-exposure (1.2 fold) and Flu-incubated PHB MPs treatment (1 fold, Figure 3D).

Discussion

The present study provides the first experimental evidence~~s~~ of ~~the~~ stress-related effects initiated~~caused~~ by novel bio-microplastic exposure, either as a single contaminant or in combination with Flu, in ~~to~~ the blue mussel *Mytilus edulis.* The comparison was conducted~~made~~ with the more traditional oil-based plastic polyethylene. Flu and both PE and PHB MPs modified the antioxidant responses in both gills and digestive glands. The present results are largely in agreement ~~line~~ with those previously reported for Flu and PE MPs, both in single or combined exposures (Magara et al., 2018).

PE MPs altered the levels of several of the tested biomarkers. Although previous studies showed that oil-based plastic led to enhanced antioxidant activities in copepods (Jeong et al., 2017) and fish (Barboza et al., 2018), our results are in agreement with studies on mussels~~This outcome is in agreement line with previous studies~~, showing a significant inhibition of CAT, SeGPx and GST activity following oil-based MPs mussel exposure (Avio et al., 2015; Paul-Pont et al., 2016). A ~~D~~ decrease of CAT and GPx activities in digestive glands and of SeGPx and GST in gills exposed to PE MPs may be related to the size and shape of microplastics, that ~~can~~ play a key role in initiation of ~~on promote~~ biological changes (Browne et al., 2008; Von Moos, 2012; Avio et al., 2015). Small polystyrene plastic particles may ~~can~~ be translocated from the gut to the circulatory system of *Mytilus edulis* and subsequently ~~be~~ retained~~,~~ suggesting that these substances y might ~~can~~ reach crucial organs such as the heart or ~~the~~ hepatic tissues to produce adverse effects~~and then hurt~~ (Browne et al., 2008). It is well known that oil-based microplastics may accumulate within organisms and induce ~~cause~~ tissue abrasions, as evidenced by histological changes in digestive cells and triggered inflammatory responses, formation of granulocytomas and lysosomal destabilization (Avio et al., 2015; Von Moos, 2012). Therefore, in this scenario, the imbalance of antioxidant defense markers might be expected ~~is not surprising~~ and ~~can~~ might ~~perhaps~~ be attributed to ~~the~~ physical damage induced by MPs enabling further accumulation of reactive products. ~~leading to a slight accumulation of reactive products.~~ Further~~more~~, in the present study ~~the~~ increased GR activity in digestive glands might reflect the ~~can sustenance the rising~~ demand for ~~of~~ GSH as defense mechanism against oxidative stress induced ~~caused~~ by oil-based MPs (Jeong et al., 2016).

~~THIS IS RESULTS! DELETEResults from this study described, for the first time, that exposure to bioplastic PHB MPs also showed impairment of antioxidant biomarkers. Activity of SOD decreased only in digestive glands, CAT in gills and Se-GPx in both tissues.~~

~~NO EVIDENCE FOR INFLAMMATION DELETEAs for PE MPs, we may hypothesize a mechanic hurt, following inflammatory response. However, because of the biodegradability potential of PHB, chemical damages cannot be excluded. Indeed, it is feasible that potential toxic compounds have been generated during biopolymer degradation process. At our best knowledge, no studies have been carried out on the metabolism of PHB in marine mussels.METABOLISM NOT MEASURED Though, it is known that the PHB degradation rate is in the order of few months in anaerobic environment, and years in seawater (Madison and Huisman, 1999; Verlinden et al., 2007). Within the organisms, PHB has a degradation rate, albeit low, due to its physical-chemical characteristics, such as the high crystallinity (Zinn et al., 2001; Anjum et al., 2016). Therefore, we may suppose that during the 96h trial, *Mytilus edulis* has start to degraded PHB MPs and a higher hydroxybutyrate tissue concentration occurred,~~

~~leading to a triggered ROS production in combination with physical damage.YOU DID NOT MEASURE THIS – DELETE~~

The present study also carried out a co-exposure and incubation scenario of both PE and PHB MPs with Flu and reported~~the results have provided~~ information on oxidative stress related to the simultaneous exposure to compounds. In this scenario, a mussel experimental group exposed to Flu-only was used as positive control. Previously Pan et al (2005) found that at high benzo(k)fluoranthene, a PAH, concentrations the activity of antioxidant enzymes ~~WHICH STATE??~~ was diminished in the scallop *Chlamys farreri* in a ~~The~~ time-dependent manner. ~~course tendency of antioxidant enzymes to restrain activities at high benzo(k)fluoranthene concentrations was already reported on scallop *Chlamys farreri* (Pan et al., 2005). Accordingly,~~ Our findings that the marked decrease of SOD activity levels and both GPx~~'s~~ in digestive glands, as well as ~~of~~ SeGPx ~~also~~ in gills and CAT ~~catalase~~ activity in both tissues indicate ~~suggests~~ that this Flu ~~PAH~~ may exert detrimental effects when ~~singularly~~ administered alone resulting in~~, causing~~ a severe oxidative stress ~~o~~in *Mytilus edulis*. The impairment of antioxidant biomarkers levels may be attributed to a chemical-mediated damage. Indeed, the enhanced ~~triggered~~ GST activity measured in digestive glands might represent an important outcome, since this ~~may~~ indicate elevated~~a strengthening~~ of phase-II biotransformation metabolism. The significant induction of GST activity in mussels exposed to Flu-only may~~can~~ be attributed ~~explained~~ ~~due~~ to ~~the~~ production of specific substrates for GST by phase I enzymes, representing an effective defense line against Flu.~~fluoranthene.~~ Babson et al (1986) showed that rat liver microsomes converted Flu to trans-2,3-dihydrodiol as the major metabolite. ~~Metabolism study on rat liver microsomes showed that Flu is converted to trans-2,3-dihydrodiol as the major metabolite (Babson et al., 1986)~~. Although data regarding ~~about~~ Flu metabolites in blue mussels are ~~is~~ lacking, ~~we can~~ it is suggested that GST induction may be related to ~~the~~ production of this intermediate.

~~However,~~ ~~b~~Biomarkers responses in digestive glands and gills of mussels exposed to both Flu and PE or PHB MPs (co-exposure and incubated treatment) generally followed a trend similar to ~~the~~ PAH alone ~~own~~ ~~traditional~~ ~~and~~ or the new plastic alone and no combined effects of these agents was apparent. The absence of combined effects in *Mytilus edulis* exposed to PE MPs and Flu is in agreement ~~consistent~~ with ~~the results of our previous study~~ our previous results (Magara et al., 2018)~~.~~ ~~This particular outcome may could be related to a MAKES NO SENSE retention mechanism?? of Flu exerted by PE MPs, due to MAKES NO SENSE specific physical properties~~, suggesting that MPs may act as a "sink" of environmental pollutants (Chua et al. 2014; Khan et al. 2015). Chua et al. (2014), showed that the concentration of ~~P~~polybrominated ~~D~~diphenyl ~~E~~ethers (PBDE) was lower in *Allorchestes compressa* co-exposed to microplastics then specimens treated with ~~single~~ PBDE alone. ~~The authors~~ Chua et al (2014) concluded that the presence of MPs may inhibit the uptake of PBDE, perhaps because this contaminant is strongly absorbed onto microplastics surface~~,~~ making it less bioavailable. ~~(Chua et al., 2014).~~ It is already known that oil-based micropolymers have the propensity to aggregate in water (Alimi et al., 2018) ~~(NEED REF??)~~. ~~A p~~Previously ~~study~~ Khan et al (2015) found ~~o~~in zebrafish ~~showed~~ a decrease in silver (Ag) uptake by fish attributed to ~~, probably due~~ ~~to~~ diminished ~~a minimization~~ ~~of the~~ contact~~s~~ between ~~the~~ Ag and tissues following oil-based MP aggregation. ~~(Khan et al., 2015).~~ Further~~more~~, Magara et al. (2018) reported that Flu tissue~~s~~ concentrations were ~~as~~ lower in *Mytilus edulis* exposed to both co-exposure with ~~and incubated~~ polyethylene and PAH compared to specimens treated with Flu alone. ~~fluoranthene only~~. The absence of combined effects of PE MPs and Flu in *Mytilus edulis* ~~exposed to~~ ~~PE MPs, either in co-exposure~~ ~~or incubated treatment,~~ and previous results on Flu uptake in ~~B~~blue mussel (Magara et al., 2018), suggests that the interaction of tissues with Flu might ~~could~~ be delayed by this aggregation mechanism exerted by microplastics.

~~THIS WAS NOT MEASURED, DELETE~~ ~~Concerning the biodegradable PHB polymer, biomarkers responses surprisingly allow to suppose characteristics similar to polyethylene, although knowledge on~~

~~PHB retention or aggregation properties are not available However, due to its ability to degrade in tissues, further studies are needed to gather knowledge on the pro-oxidant role of bio-microplastic.~~

~~The results of the present research show for the first time a role of bioplastics in affecting antioxidant and THIS WAS NOT DONEdetoxifying pathways.~~

Bioplastics are currently becoming the leading material for replacing oil-based polymers since it is more desirable than traditional plastic due to a ~~its~~ propensity to biodegrade in the environment (Anjum et al., 2016). ~~A~~ ~~r~~Recently Napper and Thompson (2019) ~~study~~ demonstrated that biodegradable polymers may ~~could~~ not undergo any substantial deterioration over a 3 year period in marine environment, but may be reduced in small fragments~~, as is~~ similar to ~~for~~ oil-based plastics~~ (Napper and Thompson, 2019)~~. Our data suggest that PHB MPs results in ~~may~~ ~~lead to~~ altered levels of some oxidative stress biomarkers~~, again which is~~ similar to oil-based MPs. The fact that bio-microplastics may exert stress effects and the potential low biodegradability rate ~~may~~ indicate a novel threat for the marine environment. Therefore, it is crucial to understand ~~the~~ degradation processes of bioplastics in the environment, but ~~whilst~~ also gathering knowledge regarding ~~about~~ ~~their~~ potential ecotoxicological impacts. ~~Whilst~~ Although the damage exerted by oil-based polymers is becoming increasingly understood, it is still ~~now~~ necessary to investigate the possible consequences ~~impacts~~ of bioplastics exposure~~,~~ both alone and in combination with other environmental pollutants present in the aquatic ecosystem.

Conclusions

The results of the present study demonstrated ~~suggest~~ that PHB MPs ~~may~~ modify the baseline levels of biomarkers related to oxidative stress in *Mytilus edulis*. These ~~levels of~~ alterations were similar to those exerted by PE MPs treatments for some~~few~~ of the antioxidant ~~detoxifying~~ biomarkers. Further~~more~~, the responses measured in both co-treatments were similar to those noted with ~~of~~ MP-alone~~only~~ exposures. Data indicated ~~This suggests~~ the absence of any ~~no~~ combined effects produced

~~caused~~ by oil-based MPs or bioplastics MPs and Flu. ~~fluoranthene. Further studies are required to gather information on the potential consequences of new and innovative bioplastic polymer in the environment.~~

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Legend of Figures

Figure 1. SOD (A and B) and CAT (C and D) activity in gills (A and C) and digestive gland (B and D) of *Mytilus edulis* exposed to different treatment groups A-H as follows: (A) Control (no added contaminants), (B) Flu only, (C) PE MPs only, (D) PHB MPs only, (E) PE MPs-Flu co-exposure, (F) PHB MPs-Flu co-exposure, (G) Flu-incubated PE MPs and (H) Flu-incubated PHB MPs. Bars show mean values ± S.D. Different letters (a, b, c) indicate statistical significant differences ($P < 0.05$, one-way ANOVA with post-hoc Bonferroni test). Note different scales on different x-axes.

Figure 2. GPx (A and B) and SeGPx (C and D) activity in gills (A and C) and digestive gland (B and D) of *Mytilus edulis* exposed to different treatment groups A-H as follows: (A) Control (no added contaminants), (B) Flu only, (C) PE MPs only, (D) PHB MPs only, (E) PE MPs-Flu co-exposure, (F)

PHB MPs-Flu co-exposure, (G) Flu-incubated PE MPs and (H) Flu-incubated PHB MPs. Bars show mean values ± S.D. Different letters (a, b, c) indicate statistical significant differences ($P < 0.05$, one-way ANOVA with post-hoc Bonferroni test). Note different scales on different x-axes.

Figure 3. GST (A and B) and GR (C and D) activity in gills (A and C) and digestive gland (B and D) of *Mytilus edulis* exposed to different treatment groups A-H as follows: (A) Control (no added contaminants), (B) Flu only, (C) PE MPs only, (D) PHB MPs only, (E) PE MPs-Flu co-exposure, (F) PHB MPs-Flu co-exposure, (G) Flu-incubated PE MPs and (H) Flu-incubated PHB MPs.

Bars show mean values ± S.D. Different letters (a, b, c) indicate statistical significant differences ($P < 0.05$, one-way ANOVA with post-hoc Bonferroni test).

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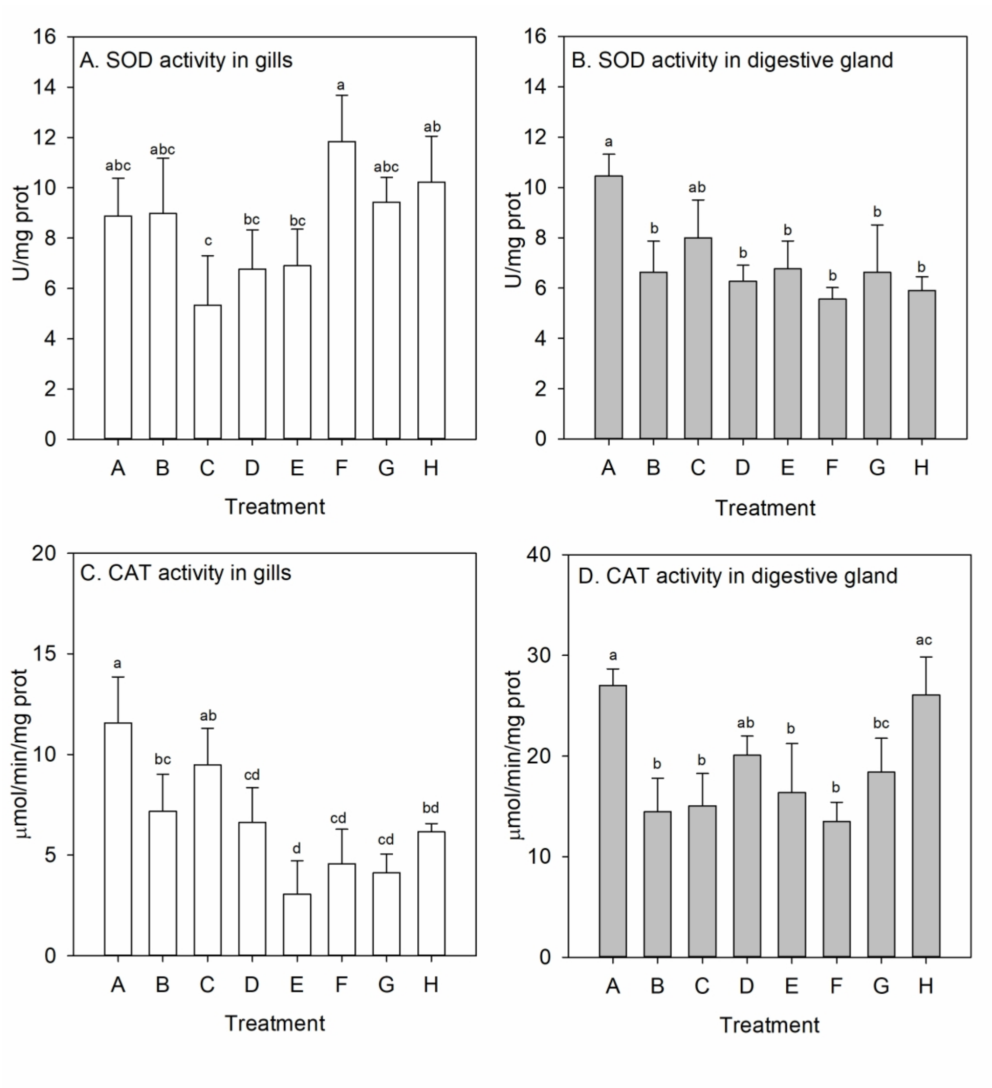

Figure 1

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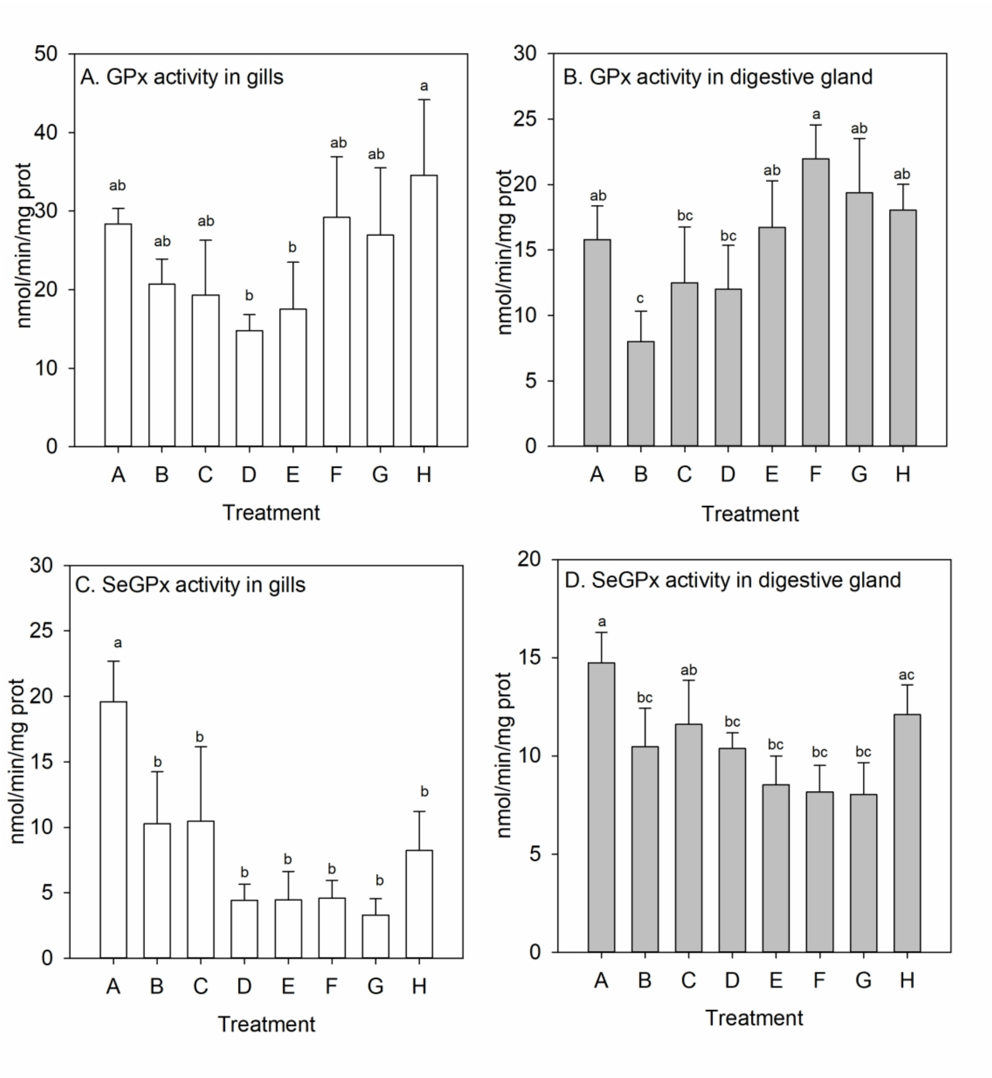

Figure 2

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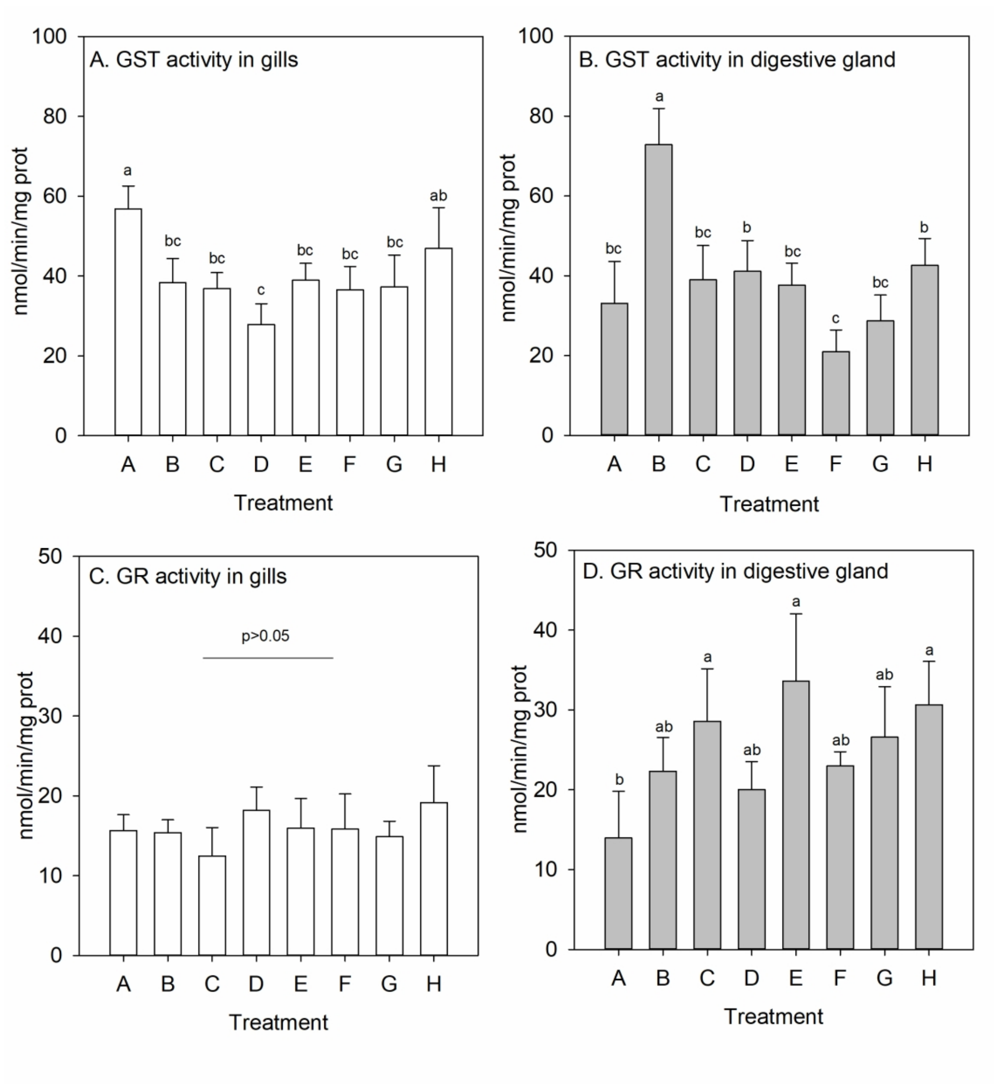

Figure 3

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